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APPLICATION NUMBER: ***60/434,974***
FILING DATE: ***December 20, 2002***
RELATED PCT APPLICATION NUMBER: ***PCT/US03/41808***

THE COUNTRY CODE AND NUMBER OF YOUR PRIORITY APPLICATION, TO BE USED FOR FILING ABROAD UNDER THE PARIS CONVENTION, IS ***US60/434,974***

Certified by

Jon W. Dudas

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PTO/SB/16 (10-01)
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

Express Mail Label No. ET860318380US

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
RICHARD	EBERSOLE	WILMINGTON, DELAWARE
LINDA J.	SCHWARTZ	NEWARK, DELAWARE
RAYMOND E.	JACKSON	NEWARK, DELAWARE

☐ *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

SEQUENCES DIAGNOSTIC FOR FOOT AND MOUTH DISEASE

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 31 ☐ CD(s), Number

☒ Drawing(s) *Number of Sheets* 5 ☒ Other (specify) SEQUENCE LISTING – 5 PGS, FEE TRANSMITTAL

☐ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☐ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check or money order is enclosed to cover the filing fees

FILING FEE AMOUNT ($)

☒ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 04-1928 — 160.00

☐ Payment by credit card. Form PTO-2038 is attached.

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____.

Respectfully submitted,

SIGNATURE _____

TYPED or PRINTED NAME NEIL FELTHAM

TELEPHONE 302-992-4946

Date 12/20/02

REGISTRATION NO. (If appropriate) 36,506

Docket Number CL2272 US PRV

60439974 v1 120202

PTO/SB/17 (10-02)
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JC525 U.S. PTO 12/20/02

FEE TRANSMITTAL for FY 2003

Patent fees are subject to annual revision.

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 160.00

Complete if Known	
Application Number	UNKNOWN
Filing Date	DECEMBER 20, 2002
First Named Inventor	EBERSOLE ET AL.
Examiner Name	UNKNOWN
Group / Art Unit	UNKNOWN
Attorney Docket No.	CL2272 US PRV

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☒ Deposit Account:

Deposit Account Number: 04-1928

Deposit Account Name: E. I. du Pont de Nemours and Company

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	740	2001	370	Utility filing fee	
1002	330	2002	165	Design filing fee	
1003	510	2003	255	Plant filing fee	
1004	740	2004	370	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	160

SUBTOTAL (1) ($) 160.00

2. EXTRA CLAIM FEES

			Extra Claims		Fee from below		Fee Paid
Total Claims		-20 ** =	0	X	18	=	0
Independent Claims		-3 ** =	0	X	84	=	0
Multiple Dependent	☐			X	280	=	

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	84	2201	42	Independent claims in excess of 3
1203	280	2203	140	Multiple dependent claim, if not paid
1204	84	2204	42	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0.00

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FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet.	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	400	2252	200	Extension for reply within second month	
1253	920	2253	460	Extension for reply within third month	
1254	1,440	2254	720	Extension for reply within fourth month	
1255	1,960	2255	980	Extension for reply within fifth month	
1401	320	2401	160	Notice of Appeal	
1402	320	2402	160	Filing a brief in support of an appeal	
1403	280	2403	140	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive – unavoidable	
1453	1,280	2453	640	Petition to revive – unintentional	
1501	1,280	2501	640	Utility issue fee (or reissue)	
1502	460	2502	230	Design issue fee	
1503	620	2503	310	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17 (q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	740	2809	370	Filing a submission after final rejection (37 CFR § 1.129(a))	
1810	740	2810	370	For each additional invention to be examined (37 CFR § 1.129(b))	
1801	740	2801	370	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify)

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($) 0.00

SUBMITTED BY				Complete *(if applicable)*	
Name (Print/Type)	NEIL FELTHAM	*Registration No. (Attorney/Agent)*	36,506	*Telephone*	302-992-6460
Signature				*Date*	DECEMBER 20, 2002

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This collection of information is required by 37 CFR 1.17 and 1.27. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducint this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office,

TITLE

SEQUENCES DIAGNOSTIC FOR FOOT AND MOUTH DISEASE

FIELD OF THE INVENTION

The invention relates to the field of diagnostic testing. More specifically, new primers have been isolated that are diagnostic for the Foot and Mouth Disease Virus (FMDV).

BACKGROUND OF THE INVENTION

Recent events in the United Kingdom have demonstrated very clearly that foot and mouth disease virus(FMDV) is so highly contagious that rapid diagnosis is required to control its spread. See, i.e., Adam, D., *Nature* 410:398 (2001) and Enserink, M., *Science* 291:2298-2300 (2001).

Foot and Mouth Disease Virus (FMDV) is actually a group of closely related viruses, classified as members of the genus Aphthovirus and family Picornaviridae. The genus aphthovirus has two members, FMDV and Equine Rhinitis A Virus (ERV-1). The second genus member, ERV-1, shares some sequence homology with FMDV, but is not a cause of foot and mouth disease (FMD). ERV- 1 is the agent of an equine respiratory disease (horses are not susceptible to FMDV). The seven serotypes of FMDV include types A, O. C, Asia 1, Sat 1 (South African Territories), Sat 2, and Sat 3. Serotypes are distinguishable by serotype specific enzyme linked immunosorbent assays (ELISA). Because of the range of species affected, the high rate of infectivity, and the fact that FMDV is shed before clinical signs occur, FMD is one of the most feared reportable diseases known in North America. Disease caused by FMDV is devastating to farm animals and can have a major economic impact on countries producing cloven hoved animals (cattle, pigs, sheep, goats and camelids) or their products. Clearly, new and more sensitive assays for the detection of this disease are needed.

A variety of methods for the detection of FMDV have been developed. These fall into three general categories: 1.) detection of FMDV peptides; 2.) detection of FMDV generated antibodies; and 3.) detection of FMDV genetic material.

A number of peptides have been identified that are unique to the FMDV and are considered diagnostic for its presence. These include both structural proteins as well as non-structural proteins (see for example Yi et al., U.S. 6,048,538; Saeki et al., U.S. 5,639,601).

In other cases methods have been developed to detect antibodies generated by the infected animal to the FMDV. The ELISA assay is the

most preferred format (see for example Gilles et al., *J. Virological Methods* 107(1):89-98 (2003); Mackay et al., *J. Virological Methods* 97(1-2):33-48 (2001); Bergmann et al., *Archives of Virology* 145(3):473-489 (2000); and Ferris, N. P., Towards Livestock Disease Diagnosis and Control in the 21st Century, Proceedings of an International Symposium on Diagnosis and Control of Livestock Diseases Using Nuclear and Related Techniques, Vienna, Apr.7-11, 1997 (1998), Meeting Date 1997, 65-77; International Atomic Energy Agency:Vienna, Austria).

A common and effective method of assay has been the use of primer directed nucleic amplification methods for the amplification of diagnostic portions of the FMDV genome. These methods are based on the isolation of primers or probes that are particularly diagnostic for the presence of the virus. Collins et al. (*Biochemical and Biophysical Research Communications* 297(2):267-274 (2002)) teach an isothermal method of nucleic acid sequence-based amplification using primers based on a variety of loci in the FMDV genome. One of the most popular methods for detection is the use of a method involving reverse transcription followed by polymerase chain reaction (RT-PCR). As its name implies, the method involves the synthesis of RNA by reverse transcription and then the amplification of DNA by PCR. Callahan et al. (WO 02/095074) use this method for the detection of FMDV using primers derived from highly conserved regions of the 3D coding region of the genome. Reid et al. (*J. Virological Methods* 105(1):67-80 (2002)) teach a fluorogenic RT-PCR assay using a primer/probe set designed from the internal ribosomal entry site region of the virus genome that was capable of detecting all seven serotypes of the FMDV. The primer-based methods are amenable to a variety of formats and kits (see for example (Callahan et al., *J. American Veterinary Medical Association* 220(11):1636-1642 (2002)).

All of the above methods are useful in the detection of FMDV; however, all suffer from lack or specificity and sensitivity. Additionally, because of the high gene mutation rate in the virus, tests directed to different regions of the genome would be useful. There is a need therefore for a highly sensitive assay for FMDV that is rapid, accurate and easily used in the field. Applicants have solved the stated problem by the discovery of primers based on a new portion of the FMDV genome that may be used in primer directed amplification or nucleic acid hybridization

assay methods.

SUMMARY OF THE INVENTION

The present invention provides an isolated FMDV diagnostic primer sequence as set forth in any one of SEQ ID NOs:16-20 or an isolated nucleic acid molecule that is completely complementary to SEQ ID NOs:16-20.

Additionally the invention provides a kit for the detection of a FMDV infection in an animal comprising a pair of nucleic acid molecules of the invention.

Similarly the invention provides a vector comprising the nucleic acid molecule(s) of the invention. In a preferred embodiment the vector may be contained in a cell.

In an alternate embodiment the invention provides a method for detecting the presence of FMDV in a sample comprising:

(i) extracting RNA from a cell suspected of containing the FMDV;

(ii) probing the extracted RNA with a probe derived from the isolated FMDV diagnostic primer sequence of the invention under suitable hybridization conditions;

wherein the identification of a hybridizable nucleic acid fragment confirms the presence of FMDV.

Similarly the invention provides a method for detecting the presence of FMDV in a sample comprising:

(i) extracting RNA from a sample suspected of containing FMDV; and

(ii) amplifying the extracted RNA with at least one oligonucleotide primer corresponding to at least a portion of the isolated FMDV diagnostic primer sequence of the invention such that amplification products are generated;

wherein the presence of amplification products confirms the presence of FMDV.

In another embodiment the invention provides a method for detecting the presence of FMDV in a sample comprising:

(i) extracting total cellular RNA from a sample suspected of being infected with FMDV;

(ii) synthesizing complementary DNA strands to the extracted RNA using a reverse transcriptase and at least one oligonucleotide primer pair corresponding to at least a portion of the isolated FMDV diagnostic primer sequence of the invention;

(iii) amplifying the newly generated complementary DNA strands to the extracted RNA using at least one oligonucleotide primer corresponding to at least a portion of the isolated FMDV diagnostic primer sequence of the invention such that amplification products are generated;

wherein the presence of amplification products confirms the presence of FMDV.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE DESCRIPTIONS

Figure 1 is the sequence of synthetic DNA target (SEQ ID NO:21) used for FMD Test.

Figure 2 is a plasmid map showing the synthetic FMD DNA construct.

Figure 3 shows results from RT-PCR reactions. Specifically, Figure 3A shows RT-PCR product obtained using primers P2Fwd-10 and P33-4 and serial log dilutions of the synthetic FMD target RNA from 10^7 copies to 10^1 copies/ test. Figure 3B shows the RT-PCR response using the P2Fwd-10 and P33-4 primers with a representative strain for each of the seven FMD viral serotypes at 10^2 viral RNA copies/ test.

Figure 4 is a composite picture of three agarose gels showing the RT-PCR products formed to serotype O_{Taiwan} RNA using the P2Fwd-10 primer in combination with P33-4, LJS1 and LJS2 primers.

Figure 5 is an agarose gel showing the RT-PCR products formed using synthetic FMD RNA using the P2Fwd-10 primer in combination with P33-4 or P33 + primers.

The invention can be more fully understood from the following detailed description and the accompanying sequence descriptions, which form a part of this application.

The following sequences conform with 37 C.F.R. 1.821-1.825 ("Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures - the Sequence Rules") and consistent with World Intellectual Property Organization (WIPO) Standard

ST.25 (1998) and the sequence listing requirements of the EPO and PCT (Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the Adminstrative Instructions). The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

SEQ ID NOs:1-13 are linkers for construction of synthetic FMD DNA.

SEQ ID NO:14 and SEQ ID NO:15 encodes primers Amplicon 5' and Amplicon 3'.

SEQ ID NO:16 is the nucleotide sequence of a 5' Forward diagnostic primer, P2-Fwd2, which binds to 3903-3929 bp of GenBank AF308157.

SEQ ID NO:17 are is the nucleotide sequence of a 3' Reverse diagnostic primer, P33-4, which binds to 4086-4108 bp of GenBank AF308157.

SEQ ID NO:18 are is the nucleotide sequence of a 3' Reverse diagnostic primer, P33+, which binds to 4083-4111 bp of GenBank AF308157.

SEQ ID NO:19 are is the nucleotide sequence of a 3' Reverse diagnostic primer, LJS1, which binds to 4460-4489 bp of GenBank AF308157.

SEQ ID NO:20 are is the nucleotide sequence of a 3' Reverse diagnostic primer, LJS2, which binds to 4317-4341 bp of GenBank AF308157.

SEQ ID NO:21 is the synthetic FMD target, as shown in Figure 1.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to the identification of primers useful in assays for the detection of the Foot and Mouth Disease. The primers have been used in DNA amplification as well as hybridization assays for the efficient detection of virulent Foot and Mouth Virus.

Definitions

In this disclosure, a number of terms and abbreviations are used. The following definitions are provided.

"Polymerase chain reaction" is abbreviated PCR.

"Foot and Mouth Disease Virus" is abbreviated FMDV.

"Foot and Mouth Disease" is abbreviated FMD.

"Reverse transcription followed by polymerase chain reaction" is abbreviated RT-PCR.

The term "isolated FMDV diagnostic primer sequence" refers to a sequence corresponding to a portion of the FMDV genome being diagnostic for the presence of FMDV.

As used herein, an "isolated nucleic acid fragment" is a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

The term "amplification product" refers to portions of nucleic acid fragments that are produced during a primer directed amplification reaction. Typical methods of primer directed amplification include polymerase chain reaction (PCR), ligase chain reaction (LCR) or strand displacement amplification (SDA). If PCR methodology is selected, the replication composition would include, for example: nucleotide triphosphates, two primers with appropriate sequences, DNA or RNA polymerase and proteins. These reagents and details describing procedures for their use in amplifying nucleic acids are provided in U.S. Patent No. 4,683,202 (1987, Mullis, et al.) and U.S. Patent No. 4,683,195 (1986, Mullis, et al.). If LCR methodology is selected, then the nucleic acid replication compositions would comprise, for example: a thermostable ligase (e.g., *T. aquaticus* ligase), two sets of adjacent oligonucleotides (wherein one member of each set is complementary to each of the target strands), Tris-HCl buffer, KCl, EDTA, NAD, dithiothreitol and salmon sperm DNA. See, for example, Tabor et al., *Proc. Acad. Sci. U.S.A.*, 82:1074-1078 (1985)). Additional methods of RNA replication such as replicative RNA system (Qβ-replicase) and DNA dependent RNA-polymerase promoter systems (T7 RNA polymerase) are contemplated to be within the scope of the present invention.

The term "reverse transcription followed by polymerase chain reaction", or "RT-PCR", refers to a sensitive technique for quantitative analysis of gene expression, cloning, cDNA library construction, probe synthesis, and signal amplification in *in situ* hybridizations. The technique consists of two parts: synthesis of cDNA from RNA by reverse transcription (RT), and amplification of a specific cDNA by polymerase chain reaction (PCR). Reverse Transcriptase is an RNA dependent DNA polymerase that catalyses the polymerization of nucleotides using template DNA, RNA or RNA:DNA hybrids. It is important to utilize a total

RNA isolation technique that yields RNA lacking significant amounts of genomic DNA contamination, since the subsequent PCR cannot discriminate between cDNA targets synthesized by reverse transcription and genomic DNA contamination.

The term "primer" refers to an oligonucleotide (synthetic or occurring naturally), which is capable of acting as a point of initiation of nucleic acid synthesis or replication along a complementary strand when placed under conditions in which synthesis of a complementary stand is catalyzed by a polymerase.

The term "probe" refers to an oligonucleotide (synthetic or occurring naturally) that is significantly complementary to a "fragment" and forms a duplexed structure by hybridization with at least one strand of the fragment.

The term "replication inhibitor moiety" refers to any atom, molecule or chemical group that is attached to the 3' terminal hydroxyl group of an oligonucleotide that will block the initiation of chain extension for replication of a nucleic acid strand. Examples are, but not limited to: 3'-deoxynucleotides (e.g., cordycepin), dideoxynucleotides, phosphate, ligands (e.g., biotin and dinitrophenol), reporter molecules (e.g., fluorescein and rhodamine), carbon chains (e.g., propanol), a mismatched nucleotide or polynucleotide, or peptide nucleic acid units.

The term "non-participatory" will refer to the lack of participation of a probe or primer in a reaction for the amplification of a nucleic acid molecule. Specifically a non-participatory probe or primer is one that will not serve as a substrate for, or be extended by, a DNA or RNA polymerase. A "non-participatory probe" is inherently incapable of being chain extended by a polymerase. It may or may not have a replication inhibitor moiety.

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength. Hybridization and washing conditions are well known and exemplified in Sambrook, J., Fritsch, E. F. and Maniatis, T. Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989), particularly Chapter 11 and Table 11.1 therein (entirely incorporated herein by reference). The

conditions of temperature and ionic strength determine the "stringency" of the hybridization. For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a T*m* of 55°, can be used, e.g., 5X SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5X SSC, 0.5% SDS. Moderate stringency hybridization conditions correspond to a higher T*m*, e.g., 40% formamide, with 5X or 6X SSC. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T*m* for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T*m*) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T*m* have been derived (see Sambrook et al., *supra*, 9.50-9.51). For hybridizations with shorter nucleic acids, i.e., oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook et al., *supra*, 11.7-11.8). In one embodiment the length for a hybridizable nucleic acid is at least about 10 nucleotides. Preferable a minimum length for a hybridizable nucleic acid is at least about 15 nucleotides; more preferably at least about 20 nucleotides; and most preferably the length is at least 30 nucleotides. Furthermore, the skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as necessary according to factors such as length of the probe.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived

from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the invention. Expression may also refer to translation of mRNA into a polypeptide.

The terms "plasmid", "vector" and "cassette" refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. "Transformation cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitate transformation of a particular host cell. "Expression cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that allow for enhanced expression of that gene in a foreign host.

The term "sequence analysis software" refers to any computer algorithm or software program that is useful for the analysis of nucleotide

or amino acid sequences. "Sequence analysis software" may be commercially available or independently developed. Typical sequence analysis software will include but is not limited to the GCG suite of programs (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI), BLASTP, BLASTN, BLASTX (Altschul et al., *J. Mol. Biol.* 215:403-410 (1990), DNASTAR (DNASTAR, Inc., Madison, WI), and Vector NTi version 7.0. Within the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values" will mean any set of values or parameters which originally load with the software when first initialized.

Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989) (hereinafter "Maniatis"); and by Ausubel, F. M. et al., Current Protocols in Molecular Biology, published by Greene Publishing Assoc. and Wiley-Interscience (1987).

The Foot and Mouth Disease Virus Genome

The FMDV genome (approximately 7-9 kB) consists of a single RNA positive strand encoding four structural proteins termed VP1, VP2, VP3, and VP4, and at least 10 non-structural proteins. The non-structural proteins are encoded within sections of the genome referred to as P2 and P3. These sections can be further divided into regions 2A, 2B, and 2C, and 3A, 3B, 3C, and 3D, respectively. Various combinations of these regions encode proteins involved in viral replication. The principle viral replicase gene is located in the region known as 3D, which is about 1.5 kB in size.

Although seven distinct serotypes of FMDV have been identified to date, variations within each serotype have also been identified. Portions of many of these better known and studied variations have been sequenced; additionally, the complete genome sequence is available for the several serotypes and variations. See for example:

1. Foot-and-mouth disease virus O genomic RNA, isolate O1Campos, complete genome (AJ320488); Pereda,A.J., et al. *Arch. Virol.* 147 (11): 2225-2230 (2002);

2. Foot-and-mouth disease virus SAT 2, complete genome (NC003992);
3. Foot-and-mouth disease virus C, complete genome (NC002554); Baranowski,E., et al., *J. Virol.* 72 (8): 6362-6372 (1998);
4. Foot-and-mouth disease virus O strain China/1/99(Tibet), complete genome (AF506822);
5. Foot-and-mouth disease virus C strain C-S8 clone MARLS, complete genome (AF274010); Baranowski, E., et al. (*supra*);
6. Foot-and-mouth disease virus O, complete genome (AF308157); Beard, C.W. and Mason, P.W. *J. Virol.* 74 (2): 987-991 (2000)).

Identification of Diagnostic Region and Primer Design

The present invention provides a set of primers useful in a variety of assay formats for the highly sensitive detection of the Foot and Mouth Disease Virus (FMDV).

The 2A/2B locus of the FMD genome was selected for primer design based on the universal homology observed when multiple of the seven different serotypes were aligned using Vector NTi alignment tools. Also, the 2A/2B regions are involved in viral replication. Thus, it was reasonable to predict that these gene sequences and subsequent proteins would be conserved among the FMD serotypes, making them attractive targets for a universal RT-PCR test.

Preferred primers used herein are those that have homology to the 2A/2B locus (e.g., bp 3864-3917 and 3918-4379 of AF308157) of the FMD and include the forward or 5' primer as set forth in SEQ ID NO:16 and the three 3' reverse primers as set forth in SEQ ID NOs:17-19. One additional preferred primer is the 3' reverse primer as set forth in SEQ ID NO:20, which binds to the 2C region (e.g., bp 4380-5333 of AF308157) Details concerning these primers are shown below in Table 1.

Table 1
Primer sequences diagnostic for FMDV

Primer	SEQ ID No.	Location in FMDV Serotype O (GenBank AF308157)
P2-Fwd2, Forward	16	3903-3929
P33-4, Reverse	17	4086-4108
P33+, Reverse	18	4083-4111
LJS1, Reverse	19	4460-4489
LJS2, Reverse	20	4317-4341

These primers are broadly useful to detect FDMV infections across a plurality of serotypes and variations. Specifically, all seven serotypes of FMDV are detectable at levels < 10 $TCID_{50}$/ml and < 0.5 PFU/ml, respectively.

Assay Methods

The instant sequences may be used in a variety of formats for the detection of FMDV. The two most convenient formats will rely on methods of nucleic acid hybridization or primer directed amplification methods such as PCR.

PCR Assay Methods

In one embodiment, the present FMDV diagnostic sequences may be used as primers for use in primer directed nucleic acid amplification for the detection of the presence of FMDV. A variety of primer directed nucleic acid amplification methods are known in the art including thermal cycling methods (e.g., polymerase chain reaction (PCR) and ligase chain reaction (LCR)), as well as isothermal methods and strand displacement amplification (SDA). The preferred method is PCR. Typically, in PCR-type amplification techniques, the primers have different sequences and are not complementary to each other. Depending on the desired test conditions, the sequences of the primers should be designed to provide for both efficient and faithful replication of the target nucleic acid. Methods of PCR primer design are common and well known in the art (Thein and Wallace, "The use of oligonucleotide as specific hybridization probes in the Diagnosis of Genetic Disorders", In *Human Genetic Diseases: A Practical Approach*, K. E. Davis Ed., (1986) pp 33-50; IRL:

Herndon, VA; and Rychlik, W. (1993) In White, B. A. (ed.), Methods in Molecular Biology, Vol. 15, pp 31-39, PCR Protocols: Current Methods and Applications. Humania: Totowa, NJ).

If a nucleic acid target is to be exponentially amplified, then two primers are used each having regions complementary to only one of the stands in the target. After heat denaturation, the single-stranded target fragments bind to the respective primers which are present in excess. Both primers contain asymmetric restriction enzyme recognition sequences located 5' to the target binding sequences. Each primer-target complex cycles through nicking and polymerization/displacement steps in the presence of a restriction enzyme, a DNA polymerase and the three dNTP's and one dNTP[aS] as discussed above. An in depth discussion of SDA methodology is given by Walker et al. (*Proc. Natl. Acad. Sci. U.S.A.*, 89:392 (1992)).

Alternatively, asymmetric amplification can be used to generate the strand complementary to the detection probe. Asymmetric PCR conditions for producing single-stranded DNA would include similar conditions for PCR as described however, the primer concentrations are changed with 50 pmol of the excess primer and 1 pmol of the limiting primer. It is contemplated that this procedure would increase the sensitivity of the method. This improvement in sensitivity would occur by increasing the number of available single strands for binding with the detection probe.

Following amplification and prior to sequencing, the amplified nucleotide sequence may be ligated to a suitable vector followed by transformation of a suitable host organism with said vector. One thereby ensures a more readily available supply of the amplified sequence. Alternatively, following amplification, the amplified sequence or a portion thereof may be chemically synthesized for use as a nucleotide probe. In either situation the DNA sequence of the variable region is established using methods such as the dideoxy method (Sanger, F. et al. *Proc. Natl. Acad. Sci.*74:5463-5467 (1977)). The sequence obtained is used to guide the choice of the probe for the organism and the most appropriate sequence(s) is/are selected.

A variety of PCR detection methods are known in the art including standard non-denaturing gel electrophoresis (e.g., acrylamide or agarose), denaturing gradient gel electrophoresis, and temperature gradient gel

electrophoresis. Standard non-denaturing gel electrophoresis is the simplest and quickest method of PCR detection, but may not be suitable for all applications.

Denaturing Gradient Gel Electrophoresis (DGGE) is a separation method that detects differences in the denaturing behavior of small DNA fragments (200-700 bp). The principle of the separation is based on both fragment length and nucleotide sequence. In fragments that are the same length, a difference as little as one base pair can be detected. This is in contrast to non-denaturing gel electrophoresis, where DNA fragments are separated only by size. This limitation of non-denaturing gel electrophoresis results because the difference in charge density between DNA molecules is near neutral and plays little role in their separation. As the size of the DNA fragment increases, its velocity through the gel decreases.

DGGE is primarily used to separate DNA fragments of the same size based on their denaturing profiles and sequence. Using DGGE, two strands of a DNA molecule separate, or melt, when heat or a chemical denaturant is applied. The denaturation of a DNA duplex is influenced by two factors: 1) the hydrogen bonds formed between complimentary base pairs (since GC rich regions melt at higher denaturing conditions than regions that are AT rich); and 2) the attraction between neighboring bases of the same strand, or "stacking". Consequently, a DNA molecule may have several melting domains with each of their individual characteristic denaturing conditions determined by their nucleotide sequence. DGGE exploits the fact that otherwise identical DNA molecules having the same length and DNA sequence, with the exception of only one nucleotide within a specific denaturing domain, will denature at different temperatures or T*m*. Thus, when the double-stranded (ds) DNA fragment is electrophoresed through a gradient of increasing chemical denaturant it begins to denature and undergoes both a conformational and mobility change. The dsDNA fragment will travel faster than a denatured single-stranded (ss) DNA fragment, since the branched structure of the single-stranded moiety of the molecule becomes entangled in the gel matrix. As the denaturing environment increases, the ds DNA fragment will completely dissociate and mobility of the molecule through the gel is retarded at the denaturant concentration at which the particular low denaturing domains of the DNA strand dissociate. In practice, the

electrophoresis is conducted at a constant temperature (around 60°C) and chemical denaturants are used at concentrations that will result in 100% of the DNA molecules being denatured (i.e., 40% formamide and 7M urea). This variable denaturing gradient is created using a gradient maker, such that the composition of each DGGE gel gradually changes from 0% denaturant up to 100% denaturant. Of course, gradients containing a reduced range of denaturant (e.g., 35% to 60%) may also be poured for increased separation of DNA.

The principle used in DGGE can also be applied to a second method that uses a temperature gradient instead of a chemical denaturant gradient. This method is known as Temperature Gradient Gel Electrophoresis (TGGE). This method makes use of a temperature gradient to induce the conformational change of dsDNA to ssDNA to separate fragments of equal size with different sequences. As in DGGE, DNA fragments with different nucleotide sequences will become immobile at different positions in the gel.

Nucleic Acid Hybridization Methods

The basic components of a nucleic acid hybridization test include a probe, a sample suspected of containing cells infected with FMDV, and a specific hybridization method. Probes of the present invention are single stranded nucleic acid sequences which are complementary to the nucleic acid sequences to be detected. Probes are "hybridizable" to the nucleic acid sequence to be detected. Typically the probe length can vary from as few as 5 bases to the full length of the FMDV diagnostic sequence and will depend upon the specific test to be done. Only part of the probe molecule need be complementary to the nucleic acid sequence to be detected. In addition, the complementarity between the probe and the target sequence need not be perfect. Hybridization does occur between imperfectly complementary molecules with the result that a certain fraction of the bases in the hybridized region are not paired with the proper complementary base. A probe may be composed of either RNA or DNA. The form of the nucleic acid probe may be a marked single stranded molecule of just one polarity or a marked single stranded molecule having both polarities present. The form of the probe, like its length, will be determined by the type of hybridization test to be done.

The sample may or may not contain the FMDV. The sample may take a variety of forms, however will generally be extracted from an animal

suspected of coming in contact with the FMDV and will include, but will not be limited to: swabs from oral and nasal cavities, body fluids (e.g., blood, blood serum, urine, fecal material, saliva, cerebrospinal fluid, lymph fluid, amniotic fluid, peritoneal fluid), tissues (e.g., muscle, skin) or bone samples. Additionally, air and soil samples may be used.

The sample nucleic acid must be made available to contact the probe before any hybridization of probe and target molecule can occur. Thus the organism's DNA must be free from the cell and placed under the proper conditions before hybridization can occur. Methods of in solution hybridization necessitate the purification of the DNA in order to be able to obtain hybridization of the sample DNA with the probe. This has meant that utilization of the in solution method for detection of target sequences in a sample requires that the nucleic acids of the sample must first be purified to eliminate protein, lipids, and other cell components, and then contacted with the probe under hybridization conditions. Methods for the purification of the sample nucleic acid are common and well known in the art (Maniatis, *supra*).

Similarly, hybridization methods are well defined. Typically the probe and sample must be mixed under conditions which will permit nucleic acid hybridization. This involves contacting the probe and sample in the presence of an inorganic or organic salt under the proper concentration and temperature conditions. The probe and sample nucleic acids must be in contact for a long enough time that any possible hybridization between the probe and sample nucleic acid may occur. The concentration of probe or target in the mixture will determine the time necessary for hybridization to occur. The higher the probe or target concentration, the shorter the hybridization incubation time needed.

In one embodiment, hybridization assays may be conducted directly on cell lysates, without the need to extract the nucleic acids. This eliminates several steps from the sample-handling process and speeds up the assay. To perform such assays on crude cell lysates, a chaotropic agent is typically added to the cell lysates prepared as described above. The chaotropic agent stabilizes nucleic acids by inhibiting nuclease activity. Furthermore, the chaotropic agent allows sensitive and stringent hybridization of short oligonucleotide probes to DNA at room temperature (Van Ness and Chen, *Nucl. Acids Res.* 19:5143-5151 (1991)). Suitable chaotropic agents include guanidinium chloride, guanidinium thiocyanate,

sodium thiocyanate, lithium tetrachloroacetate, sodium perchlorate, rubidium tetrachloroacetate, potassium iodide, and cesium trifluoroacetate, among others. Typically, the chaotropic agent will be present at a final concentration of about 3M. If desired, one can add formamide to the hybridization mixture, typically 30-50% (v/v).

Alternatively, one can purify the RNA prior to probe hybridization. A variety of methods are known to one of skill in the art (e.g., phenol-chloroform extraction, IsoQuick extraction (MicroProbe Corp., Bothell, WA), and others). Pre-hybridization purification is particularly useful for standard filter hybridization assays. Furthermore, purification facilitates measures to increase the assay sensitivity by incorporating *in vitro* RNA amplification methods such as self-sustained sequence replication (see for example Fahy et al., In PCR Methods and Applications, Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1991), pp. 25-33) or reverse transcriptase PCR (Kawasaki, In PCR Protocols: A Guide to Methods and Applications, M. A. Innis et al., Eds., (1990), pp. 21-27).

Once the RNA or DNA is released, it can be detected by any of a variety of methods. However, the most useful embodiments have at least some characteristics of speed, convenience, sensitivity, and specificity.

Various hybridization solutions can be employed. Typically, these comprise from about 20 to 60% volume, preferably 30%, of a polar organic solvent. A common hybridization solution employs about 30-50% v/v formamide, about 0.15 to 1M sodium chloride, about 0.05 to 0.1M buffers, such as sodium citrate, Tris-HCl, PIPES or HEPES (pH range about 6-9), about 0.05 to 0.2% detergent, such as sodium dodecylsulfate, or between 0.5-20 mM EDTA, FICOLL (Pharmacia Inc.) (about 300-500 kilodaltons), polyvinylpyrrolidone (about 250-500 kdal), and serum albumin. Also included in the typical hybridization solution will be unlabeled carrier nucleic acids from about 0.1 to 5 mg/mL, fragmented nucleic DNA (e.g., calf thymus or salmon sperm DNA, or yeast RNA), and optionally from about 0.5 to 2% wt/vol glycine. Other additives may also be included, such as volume exclusion agents which include a variety of polar water-soluble or swellable agents (e.g., polyethylene glycol), anionic polymers (e.g., polyacrylate or polymethylacrylate), and anionic saccharidic polymers (e.g., dextran sulfate).

Nucleic acid hybridization is adaptable to a variety of assay formats. One of the most suitable is the sandwich assay format. The

sandwich assay is particularly adaptable to hybridization under non-denaturing conditions. A primary component of a sandwich-type assay is a solid support. The solid support has adsorbed to it or covalently coupled to it immobilized nucleic acid probe that is unlabeled and complementary to one portion of the DNA sequence. Probes particularly useful in the present invention are those derived from the present FMDV diagnostic sequences as set forth in SEQ ID NOs: 16-20.

The sandwich assay may be encompassed in an assay kit. This kit would include a first component for the collection of samples from an animal suspected of having contracted the FMDV and buffers for the disbursement and lysis of the sample. A second component would include media in either dry or liquid form for the hybridization of target and probe polynucleotides, as well as for the removal of undesirable and nonduplexed forms by washing. A third component includes a solid support (dipstick) upon which is fixed (or to which is conjugated) unlabeled nucleic acid probe(s) that is (are) complementary to a part of FMDV genome. A fourth component would contain labeled probe that is complementary to a second and different region of the same DNA strand to which the immobilized, unlabeled nucleic acid probe of the third component is hybridized.

In another embodiment, the instant FMDV diagnostic sequences may be used as 3' blocked detection probes in either a homogeneous or heterogeneous assay format. For example, a probe generated from the instant sequences may be 3' blocked or non-participatory and will not be extended by, or participate in, a nucleic acid amplification reaction. Additionally, the probe incorporates a label that can serve as a reactive ligand that acts as a point of attachment for the immobilization of the probe/analyte hybrid or as a reporter to produce detectable signal. Accordingly, genomic or cDNA isolated from a sample suspected of harboring the FMDV is amplified by standard primer-directed amplification protocols in the presence of an excess of the 3' blocked detection probe to produce amplification products. Because the probe is 3' blocked, it does not participate or interfere with the amplification of the target. After the final amplification cycle, the detection probe anneals to the relevant portion of the amplified DNA and the annealed complex is then captured on a support through the reactive ligand.

The instant probe is versatile and may be designed in several alternate forms. The 3' end of the probe is blocked from participating in a primer extension reaction by the attachment of a replication inhibiting moiety. Typical replication inhibitor moieties will include, but are not limited to: dideoxynuleotides, 3-deoxynucleotide, a sequence of mismatched nucleosides or nucleotides, 3' phosphate groups and chemical agents. Within the context of the present invention cordycepin (3' deoxyadenosine) is preferred.

The replication inhibitor is covalently attached to the 3' hydroxy group of the 3' terminal nucleotide of the non-participatory probe during chemical synthesis, using standard cyanoethyl phosphoramidite chemistry. This process uses solid phase synthesis chemistry in which the 3' end is covalently attached to an insoluble support (controlled pore glass, or "CPG") while the newly synthesized chain grows on the 5' terminus. Within the context of the present invention, 3-deoxyribonucleotides are the preferred replication inhibitors. Cordycepin (3-deoxyadenosine) is most preferred. Since the cordycepin will be attached to the 3' terminal end of the probe, the synthesis is initiated from a cordycepin covalently attached to CPG, 5-dimethoxytrityl-N-benzoyl-3-deoxyadenosine (cordycepin), 2-succinoyl-long chain alkylamino-CPG (Glen Research, Sterling, VA). The dimethoxytrityl group is removed and the initiation of the chain synthesis starts at the deprotected 5' hydroxyl group of the solid phase cordycepin. After the synthesis is complete, the oligonucleotide probe is cleaved off the solid support leaving a free 2' hydroxyl group on the 3'-terminally attached cordycepin. Other reagents can also be attached to the 3' terminus during the synthesis of the non-participatory probe to serve as replication inhibitors. These include, but are not limited to: other 3-deoxyribonucleotides, biotin, dinitrophenol, fluorescein, and digoxigenin. Each of these reagents are also derivatized on CPG supports (Glen Research; Clonetech Laboratories, Palo Alto, CA).

EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without

departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

GENERAL METHODS

Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989) (Maniatis); by T. J. Silhavy, M. L. Bennan, and L. W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y. (1984); and by Ausubel, F. M. et al., Current Protocols in Molecular Biology, published by Greene Publishing Assoc. and Wiley-Interscience (1987).

Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Techniques suitable for use in the following examples may be found as set out in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology: Washington, D.C. (1994)) or by Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, 2nd ed., Sinauer Associates: Sunderland, MA (1989).

Enzymes and reagents used herein were purchased from the following vendors:

* Applied Biosystems, Foster City, CA: AmpliTaq (Catalog #N808-0160), Multiscribe (Catalog #4311235); Rnase Inhibitor (Catalog #N808-0119); Buffer II (1mM Tris-HCl pH 8.3, 5 mM KCl) (Catalog #N808-0190); $MgCl_2$ (Catalog #N808-0190)
* New England Biology, Beverly, MA: EcoRI (Catalog #R0101L); Not I (Catalog #R0189L); T4 DNA Ligase (Catalog #M0202L); T4 polynucleotide kinase (Catalog #M0201L)
* Bionexus Inc., Oakland, CA: Hot Taq (Catalog #D1002HB);
* Sigma Genosys, The Woodlands, TX: Oligonucleotides;
* Qiagen, Valencia, CA: Rnase-Free Dnase Set (Catalog #79254);
* Invitrogen Life Technologies, Carlsbad, CA: Ampicillian (Catalog #11593-019); Carbenicillin (Catalog #10177-012); 2% Agarose E-gels (Cat #G6018-02); Luria Broth (LB) media (Catalog

#10855-021); Triazol LS Reagent (Catalog #10296-028); Diethylprocarbonate (DEPC) water (Catalog #10813-012)

* Sigma-Aldrich, St. Louis, MO: Bovin Serum Albumin (BSA) (Catalog #A3294); Dimethyl Sulfoxide (DMSO) (Catalog #D8418)

Additionally, test kits and reagents were purchased from the following vendors: pCR4-TOPO vector (Invitrogen Life Technologies, Catalog #45-0030); Qiagen QIAquick PCR Purification Kit (Qiagen, Catalog #28104); Qiagen Rneasy Mini Kit (Catalog #74106); Qiagen QIAprep Spin Mini Prep Kit (Catalog # 27106); RNA Transcription kit (Stratagene, Catalog #200340, Cedar Creek, TX); and TOPO TA Cloning Kit Dual Promoter (Invitrogen Life Technologies, Catalog #45-0640).

All oligonucleotide primers and linkers were synthesized by Sigma Genosys Company, The Woodlands, TX. Polymerase chain reactions and RNA quantitations were performed using a PTC-225 Peltier Thermal Cycler (MJ Research Waltham, MA) and GeneQuant *pro* (Catalog #80-2110-98; Amersham Pharmacia Biotech, Cambridge, England).

Manipulations of genetic sequences were accomplished using the suite of programs available from the Genetics Computer Group Inc. (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI). Where the GCG program "Pileup" was used the gap creation default value of 12, and the gap extension default value of 4 were used. Where the CGC "Gap" or "Bestfit" programs were used the default gap creation penalty of 50 and the default gap extension penalty of 3 were used. In any case where GCG program parameters were not prompted for, in these or any other GCG program, default values were used.

The meaning of abbreviations is as follows: "sec" means second(s), "min" means minute(s), "hr" means hour(s), "d" means day(s), "µL" means microliter(s), "mL" means milliliter(s), "L" means liter(s), "µM" means micromolar, "mM" means millimolar, "M" means molar, "mmol" means millimole(s), "µmol" mean micromole(s), "ng" means nanogram(s), "µg" means microgram(s), "mg" means milligram(s), "g" means gram(s), "mU" means milliunit(s), and "U" means unit(s).

Construction of a Synthetic RNA Target (3800-4290 bp of FMDV serotype O)

A synthetic piece of a foot and mouth virus (FMDV) RNA serotype O (GenBank Accession Number AF308157; Beard, C.W. and Mason, P.W., *J. Virology* 74(2): 987-991 (2000)) was constructed from

base 3800 to 4290. The synthetic FMD target was constructed using 13 total DNA linkers (SEQ ID NOs: 1-13) comprising both top and bottom strands (Figure 1). *NotI* and *EcoRI* sites were added to the sequence of synthetic DNA target to facilitate directional cloning of the construct behind the T7 promoter in the pCR4-TOPO vector.

Linkers were kinased, ligated and PCR amplified using primers amplicon 5' and amplicon 3' (SEQ ID NOs: 14 and 15, respectively) in accordance to published protocols with modifications (Maniatis, *supra,* pp 5.68-5.69, 1.68-1.69, 14.2-14.19).

Construction of a Synthetic DNA (3800-4290 bp of FMDV serotype O)

To construct the synthetic FMD DNA, linkers (SEQ ID NOs: 1-13) were diluted with DEPC treated water to 25 pmoles/μl. Linkers (25 pmoles of each) were combined in one tube. To this tube 10 μl of 10x T4 Kinase buffer, 100 Units of T4 Kinase, 1 mM ATP and DEPC water to 100 μl final volume was added. The reaction was incubated for 30 min at 37°C. The kinased linkers mix was heated at 95°C for 20 min in a heat block to inactivate the kinase and melt all the linkers. After the 20 min the heat block was turned off and allowed to cool, thereby facilitating proper linker annealing.

Once the linkers cooled to room temperature, the ligation reaction was set-up as follows: in a total volume of 100 μl, 85 μl of the kinased-annealed linkers, 10 μl of 10X ligase buffer, and 50 Units of Ligase were added. The reaction proceeded for 30 min at room temperature or overnight at 14°C. Following ligation, the product was amplified by PCR to add restriction sites (if necessary) and to bulk up the quanity of product available for subsequent cloning. In a 50 μl reaction 1 μl of annealed, ligated linkers were added to a PCR tube with 1X Buffer II, 3.5 mM $MgCl_2$, 250 μM dNTP, 2.5 Units Taq, and 20 pmol of forward and reverse primers. Thermocycling conditions were: 20 cycles of 95°C (30 sec), 55°C (30 sec), 72°C (30 sec), followed by a final extension at 72°C (5 min) and a hold at 4°C. The PCR product was cleaned-up with Qiagen QIAquick PCR Purification Kit. The PCR product was subsequently digested with *Not*1/*Eco*RI and cloned into pCR4-TOPO vector cut with N*ot*1/*Eco*R1.

Cloning of the Synthetic Target

The PCR product produced above was cloned using topoisomerase-cloning technology (TOPO) developed by Invitrogen. The TOPO TA Cloning Dual Promoter Kit was used for the initial cloning of the synthetic FMD piece. Putative clones were transformed into competent *E. coli* provided by the Invitrogen kit (Top10F'). *E. coli* harboring vectors (with

or without inserts) were selected for on LB media containing 50 – 100 μg/ml ampicillian or carbenicillian for vector selection. Positive clones, containing the insert, were determined by growing up individual colonies in 4 ml of LB broth supplemented with 100 μg/ml ampicillian overnight at 37°C with 230 rpm shaking. Mini-prep DNA was prepared using a QIAprep Spin Mini Prep Kit. Clones were analyzed by restriction endonuclease digest or PCR for correctness, as determined by insert size.

The final cloning step entailed removal of the synthetic FMD fragment by enzymatically removing the insert from the TOPO TA Cloning vector using *Not I* and *Eco*RI. These restriction sites (*Not*I and *Eco*RI) were added to the ends of the synthetic FMD fragment to facilitate directional cloning of the 5-prime end behind a prokaryotic T7 promoter of the pCR4-TOPO vector. The T7 promoter facilitates RNA transcription of the synthetic FMD fragment. The final synthetic FMD construct (Figure 2), was sequenced using the M13 forward and reverse primers located on either side of the T7 synthetic FMD portion of the clone; specifically, the M13 –20 Forward primer is located at 4437-4452 bp, while the M13 Reverse primer is located at 629-645 bp. The Synthetic FMD DNA is located from 36-536 bp and the T7 promoter is located at the 5'-end of the synthetic FMD DNA from 1-20 bp.

Sequencing was conducted using fluorescent BigDye terminator chemistry (Applied Biosystems, Foster City, CA 94404). The synthetic FMD DNA construct had an identical sequence to the original serotype O sequence from base 3800 to 4290.

FMD virus is positive strand RNA virus. A positive stand RNA copy of the synthetic FMD DNA molecule prepared above was synthesized by copying the FMD DNA (Figure 1) using a T7 polymerase and the Stratagene RNA transcription kit. The RNA transcripts product was then purified and used as a surrogate FMD target molecule for reverse transcription polymerase chain reaction (RT-PCR). In this process, the synthetic FMD construct was first linearized with *Eco*RI. The digested DNA was passed through a Qiagen PCR clean-up column, thus facilitating removal of restriction endonucleases and salts. The T7 polymerase included in Stratagene's RNA Transcription kit was used to synthesize RNA from the T7 promoter located adjacent to the FMD synthetic construct. Synthetic RNA was purified using Qiagen's Mini-RNA clean-up protocol including the optional 15-minute DNAse step. RNA was eluded in DEPC treated water. Molecules of synthetic FMD RNA per micro liter were

determined spectrophotometrically (GeneQuant *pro*) and log base ten serial dilutions were routinely generated for use in RT-PCR reactions.

Example 1

Demonstration RT-PCR Assay for Detection of FMD Using Synthetic RNA

A single step RT-PCR assay for the FMD target sequence was performed on the synthetic FMD RNA target using the following reagents and conditions. Each reaction was performed in a 50 µl total reaction volume.

First, a pre-reaction mix was prepared, as follows. The forward, P2Fwd-10 (SEQ ID NO:16) and reverse primers (SEQ ID NOs:17-20) were dissolved in water and added respectively to the reaction solution at concentrations of respectively at 600 nM and 2 µM per test. Buffer II (1x) was added to comprise a final concentration of 1 mM Tris-HCl pH 8.3, 5 mM KCl and 3.5 mM $MgCl_2$. Nucleotides were used at 250 µM per test. BSA was used at a final concentration of 0.6 mM per test. SYBR Green (Catalog # 517695#S7564; Molecular Probes, Eugene, OR) was added in DMSO to a final dilution of 1:40,000. Enzymes were used at 2.5 Units Taq polymerase and 1.25 Units Multiscribe reverse transcriptase per 50 µl test. The reaction solution (45 µl) was then stored on ice.

Samples containing synthetic RNA dissolved in water were added at 5 µl per reaction. The tube(s) were sealed and then thermal cycled using the following conditions:

- 50°C 10 minutes (RT step);
- 95°C 15 minutes (Taq activation step);
- 95°C 15 second (denature step);
- 71°C 1 minute (anneal and extend step);
- Repeat denature and anneal steps 35 times;
- 71°C 10 minutes;
- 4°C hold.

The RT-PCR reaction products were then analyzed using agarose gel electrophoresis using 2% E-gels. Following electrophoresis the gels were then viewed to determine the presence or absence of a correct size RT-PCR product (224 bp).

RT-PCR reactions were performed using serial log dilutions of the synthetic FMD RNA. Sample concentrations ranged from 10^7 copies to

10^1 copies/ reaction. Reactions were carried out with primers P2Fwd-10 and P33-4 (SEQ ID NO:16 and 17) and performed as described above. Figure 3A shows representative results obtained using this set of primers. Specifically, the RT-PCR product is shown using serial log dilutions of the synthetic FMD target RNA from 10^7 copies to 10^1 copies/ test. As can be seen, the primers sensitivity allows detection of 10 copies of target RNA. The center lane contains molecular weight markers (Invitrogen low molecular weight standard).

Example 2

RT-PCR Test Response Using FMD Viral Serotypes with P2Fwd-10 and P33-4 Primer Set

This example illustrates the RT-PCR assay response to representative strains of all seven FMD viral serotypes and demonstrates that all seven serotypes can be detected.

Virus samples, each containing representative strains of all seven serotypes of FMD (O, A, C, Asia1, Sat 1, Sat 2 and Sat 3) were cultivated from field samples using *in vitro* tissue culture cell lines by Gordon Ward, USDA, APHIS, Greenport, New York. Plaque forming unit (PFU) and tissue culture infectious dose ($TCID_{50}$) determinations on the cultures established the viral titers for each sample (as described in Virology, A Practical Approach. BWJ Mahy, Ed.; IRL: Oxford and Washington D.C., 1985; Chapter 2, pp 25-35).

FMD viral RNA from the samples was isolated using the Triazol LS extraction chemistry and method as outlined by the manufacturer (Invitrogen Life Technologies, Catalog #10296-028). The recovered RNA was then reconstituted in water. Seven log dilutions were made of each FMD serotype RNA extraction.

RT-PCR reactions were performed on each of the diluted RNA serotype samples using the conditions and procedure described in Example 1. Figure 3B is a photograph of an agarose electrophoresis gel showing the typical RT-PCR product formed using samples containing a 10,000-fold dilution of the original viral RNA extracts. In this experiment, 5 ul of water was used a Negative, no-virus sample. P2Fwd-10 and P33-4 primers (SEQ ID NOs:16 and 17) were used for RT-PCR with a representative strain for each of the seven FMD viral serotypes at 10^2 viral RNA copies/ test . Viral RNA copies were determined from the viral PFU/ml and $TCID_{50}$/ml culture values. The center lane contains molecular

weight markers (Invitrogen low molecular weight standard). As shown in Figure 3B, the correct size RT-PCR product was formed with each FMD viral serotype demonstrating that the test universally detects RNA from all seven serotypes.

Example 3

RT-PCR Detection Sensitivity to FMD Serotypes

The limit of test detection for each of the seven FMD viral serotypes tested using the RT-PCR assay with the P2Fwd-10/P33-4 primers (SEQ ID NOs:16 and 17) is shown in Table 2. In this example, serial dilutions of the RNA extracted from the FMD viral cultures described above were tested using the RT-PCR assay as described in Example 1. Columns 2 and 4 of the table show the FMD virus concentrations of the original tissue cultures in $TCDI_{50}$/ml and PFU/ml units. Columns 3 and 4 show the lowest detectable dose of viral RNA detected by the RT-PCR assay in $TCDI_{50}$/ml and PFU/ml units. As shown in the Table, all seven serotypes of FMD are detectable at levels < 10 $TCID_{50}$/ml and < 0.5 PFU/ml respectively.

Table 2

RT-PCR Test Sensitivity Using P2Fwd-1-/P33-4 Primer Set

FMD Serotype	Virus Conc. $TCID_{50}$/ml	RT-PCR Sensitivity $TCID_{50}$/ml	Virus Conc. PFU/ml	RT-PCR Sensitivity PFU/ml
O	8.0E+06	0.8	7.8E+06	0.02
A	1.0E+06	1	1.2E+06	0.03
C	3.0E+06	3	3.3E+06	0.05
Asia 1	8.0E+06	8	8.0E+06	0.2
Sat1	2.0E+06	2	2.3E+06	0.06
Sat2	3.0E+06	3	3.0E+06	0.08
Sat3	4.0E+06	4	3.7E+06	0.09

Example 4

RT-PCR Assay using P2Fwd-10 Forward Primer and Three Different Reverse 3' Primers Forming Larger Products

Example 4 illustrates the utility of additional primer combinations to produce RT-PCR test products of different sizes. In this example, FMD serotype O Taiwan RNA substrate was detected using the same RT-PCR conditions described in Example 1. However, in this example, the P2Fwd-10 forward primer (SEQ ID NO: 16) was used in combination with three different reverse primers: P33-4 (SEQ ID NO:17), LJS1 (SEQ ID NO:19), or LJS2 (SEQ ID NO:20).

The advantages of the P2Fwd-10/LJS1 and P2Fwd-10/LJS2 primer sets are that they form a larger product (554 bp and 400 bp, respectively) compared to P2Fwd-10/P33-4 (224 bp). Also, the products of P2Fwd-10/LJS1 and P2Fwd-10/LJS2 primer sets can act as a substrate for half-nested PCR using the P2Fwd-10/P33-4 primer set.

Seven 10-fold serial dilutions were prepared of FMD serotype O RNA extracted in Example 2. These were tested using the above primer combinations and the RT-PCR reagent concentrations and thermal cycling conditions in Example 1. Following thermal cycling, agarose gel electrophoresis was run on the reaction products and imaged. Figure 4 illustrates the reaction products formed in response to RT-PCR reactions using the three primer sets. Specifically, Figure 5 is a composite picture of three agarose gels showing the RT-PCR products formed to serotype O_{Taiwan} RNA using the P2Fwd-10 primer in combination with P33-4, LJS1 and LJS2 primers. The RNA concentration in PFU/ml used per reaction is listed above each lane. The reverse primer type and observed product size are listed to the left of the gel picture. The fourth lane contains the molecular weight markers (Invitrogen low molecular weight standard).

According to the results, each of the primer sets produced the correct product size as determined by the FMD serotype O gene sequence. LJS1 and LJS2 primers exhibited test sensitivity down to 10^2 and 10^1 copies, respectively, and P33-4 was sensitive down to 10^{-1} PFU/ml.

Example 6

RT-PCR Test Response using Various Combinations of 5' Forward and 3' Reverse Primers

This example, illustrates the utility of additional primer combinations for RT-PCR FMD detection. In this example, serial dilutions of the synthetic FMD RNA were tested from 10^7 to 10^0 copies per reaction. A negative control was used in addition to the diluted RNA to determine the reponse of the test in the absence of viral RNA. The RNA was amplified with either the P2Fwd-10/P33-4 (SEQ ID NOs: 16 and 17) or P2Fwd-10/P33+ (SEQ ID NOs: 16 and 18) primer sets. RT-PCR reactions concentration and thermal cycling conditions were the same as described in Example 1. Figures 5 shows the gel analysis of the reaction products. The RNA concentration in copies used per reaction is listed above each lane. The fifth lane contains the molecular weight markers (Invitrogen low molecular weight) standard). Both primer sets amplify amplicon RNA. The P2Fwd-10/P33-4 primer set was sensitive to sample concentrations down to 10^0 copies/reaction and the P2Fwd-10/P33+ primer set was sensitive down to down to 10^2 copies/reaction.

CLAIMS

What is claimed is:

1. An isolated FMDV diagnostic primer sequence as set forth in SEQ ID NO:16 or an isolated nucleic acid molecule that is completely complementary to SEQ ID NO:16.

2. An isolated FMDV diagnostic primer sequence as set forth in SEQ ID NO:17 or an isolated nucleic acid molecule that is completely complementary to SEQ ID NO:17.

3. An isolated FMDV diagnostic primer sequence as set forth in SEQ ID NO:18 or an isolated nucleic acid molecule that is completely complementary to SEQ ID NO:18.

4. An isolated FMDV diagnostic primer sequence as set forth in SEQ ID NO:19 or an isolated nucleic acid molecule that is completely complementary to SEQ ID NO:19.

5. An isolated FMDV diagnostic primer sequence as set forth in SEQ ID NO:20 or an isolated nucleic acid molecule that is completely complementary to SEQ ID NO:20.

6. A pair of two different nucleic acid molecules of any of claims 1-4, each of which comprises a nucleic acid of claim 5, wherein the pair is capable of priming a polymerase chain reaction that amplifies a region of nucleic acid within said portion.

7. A kit for the detection of a FMDV comprising the pair of nucleic acid molecules of claim 6.

8. A vector comprising the nucleic acid molecule of any of claims 1-5.

9. A cell containing the vector of claim 8.

10 A method for detecting the presence of FMDV in a sample comprising:

(i) extracting RNA from a sample suspected containing the FMDV;

(ii) probing the extracted RNA with a probe derived from the isolated FMDV diagnostic primer sequence of any of Claims 1–5 under suitable hybridization conditions;

wherein the identification of a hybridizable nucleic acid fragment confirms the presence of FMDV.

11. A method according to Claim 10 wherein the probe contains at replication inhibiting moiety at the 3' end.

12. A method of Claim 11 wherein the replication inhibiting moiety is selected from the group consisting of dideoxynucleotides, a sequence of mismatched nucleotides, 3' phosphate, a molecular spacer, and 3' deoxynucleotides.

13. The method of Claim 12 where in the 3' deoxynucleotide is cordycepin.

14. A method for detecting the presence of FMDV in a sample comprising:

(i) extracting RNA from a sample suspected of containing FMDV; and

(ii) amplifying the extracted RNA with at least one oligonucleotide primer corresponding to at least a portion of the isolated FMDV diagnostic primer sequence of any of Claims 1–5 such that amplification products are generated;

wherein the presence of amplification products confirms the presence of FMDV.

15. A method for detecting the presence of FMDV in a sample comprising:

(i) extracting total RNA from a sample suspected of being infected with FMDV;

(ii) synthesizing complementary DNA strands to the extracted RNA using a reverse transcriptase and at least one oligonucleotide primer corresponding to at least a portion of the isolated FMDV diagnostic primer sequence of any of Claims 1–5;

(iii) amplifying the newly generated complementary DNA strands to the extracted RNA using at least one oligonucleotide primer corresponding to at least a portion of the isolated FMDV diagnostic primer sequence of any of Claims 1–5 such that amplification products are generated;

wherein the presence of amplification products confirms the presence of FMDV.

16. A method according to either Claim 14 or 15 the FMDV belongs to a serotype selected from the group consisting of A, O. C, Asia 1, Sat 1 Sat 2, and Sat 3.

CI2272 US PRV

TITLE

SEQUENCES DIAGNOSTIC FOR FOOT AND MOUTH DISEASE

ABSTRACT OF THE DISCLOSURE

Primers have been isolated that are diagnostic for the detection of the foot and mouth disease virus. These primers are able to detect all seven serotypes of FMD at levels < 10 $TCID_{50}$/ml and < 0.5 PFU/ml, respectively.

SNF/dmm

Figure 1

Not1 Site

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EcoRI site

(SEQ ID NO:21)

Figure 2

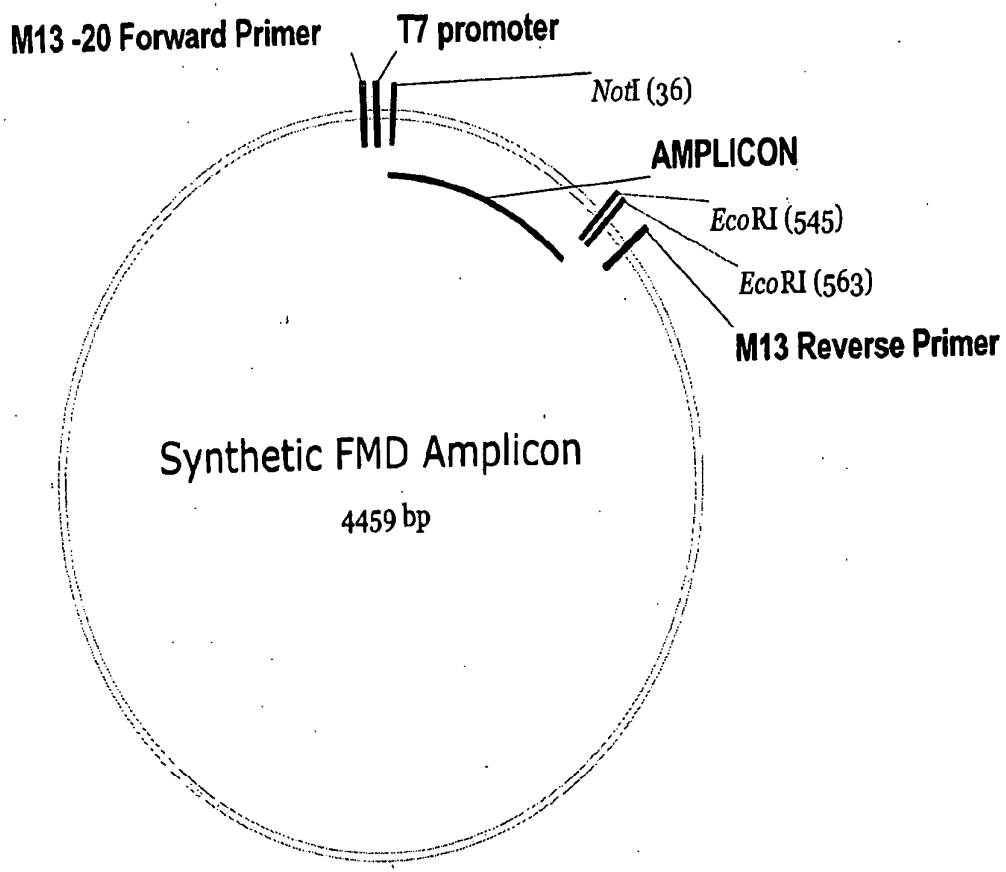
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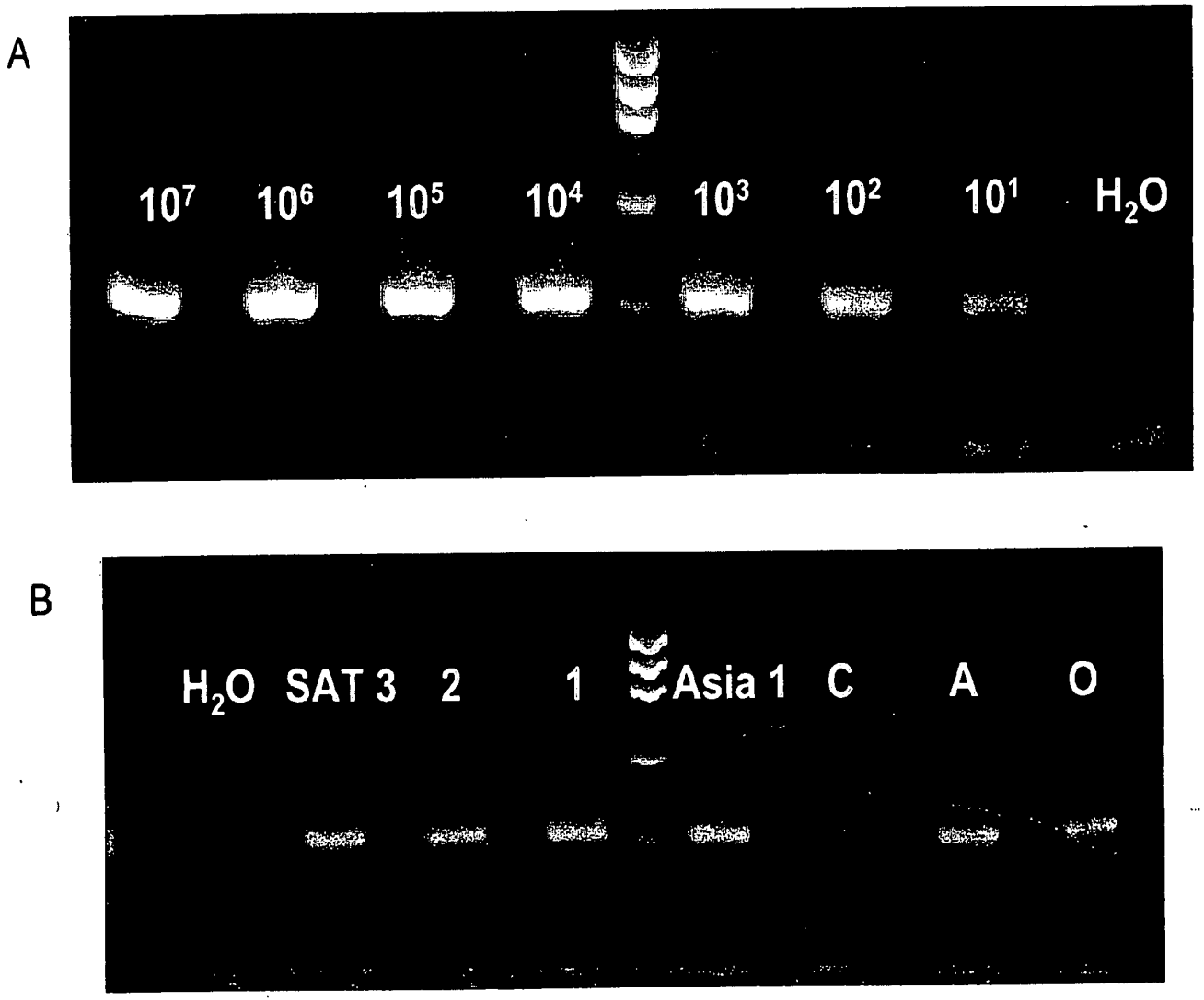
Figure 3



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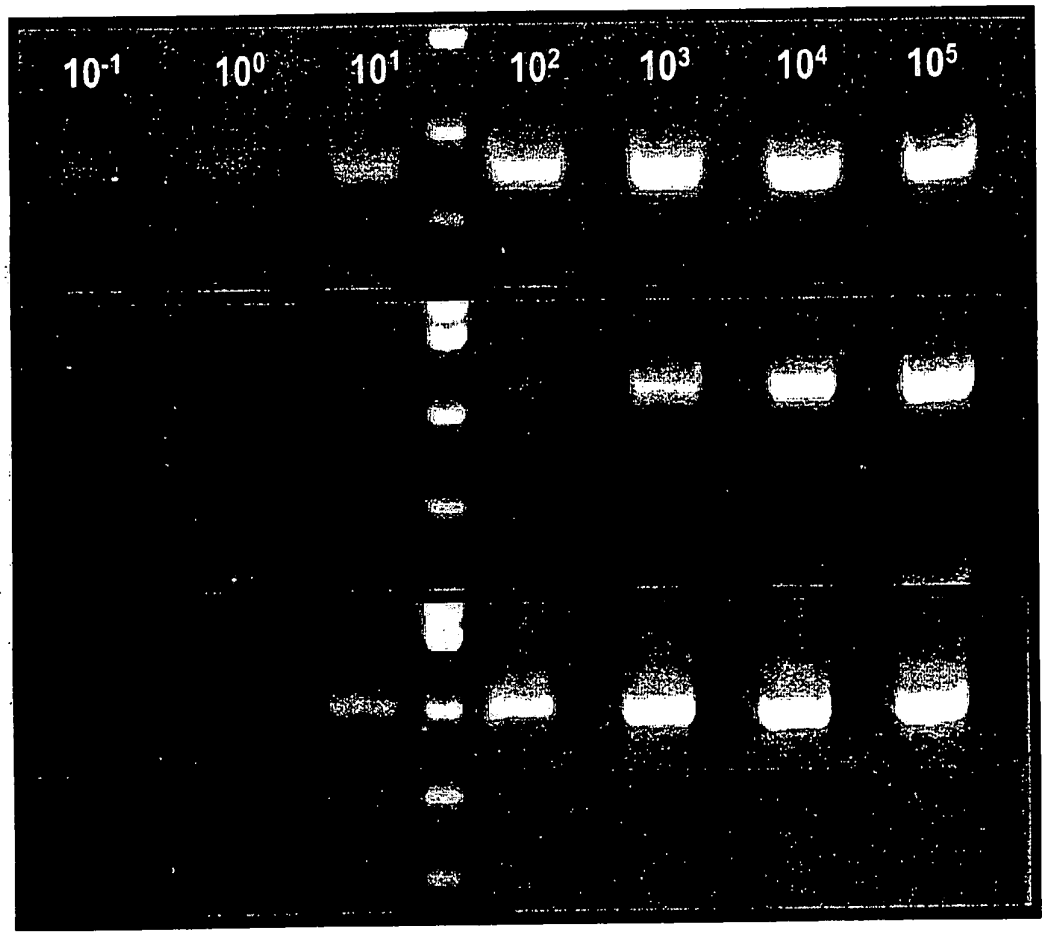

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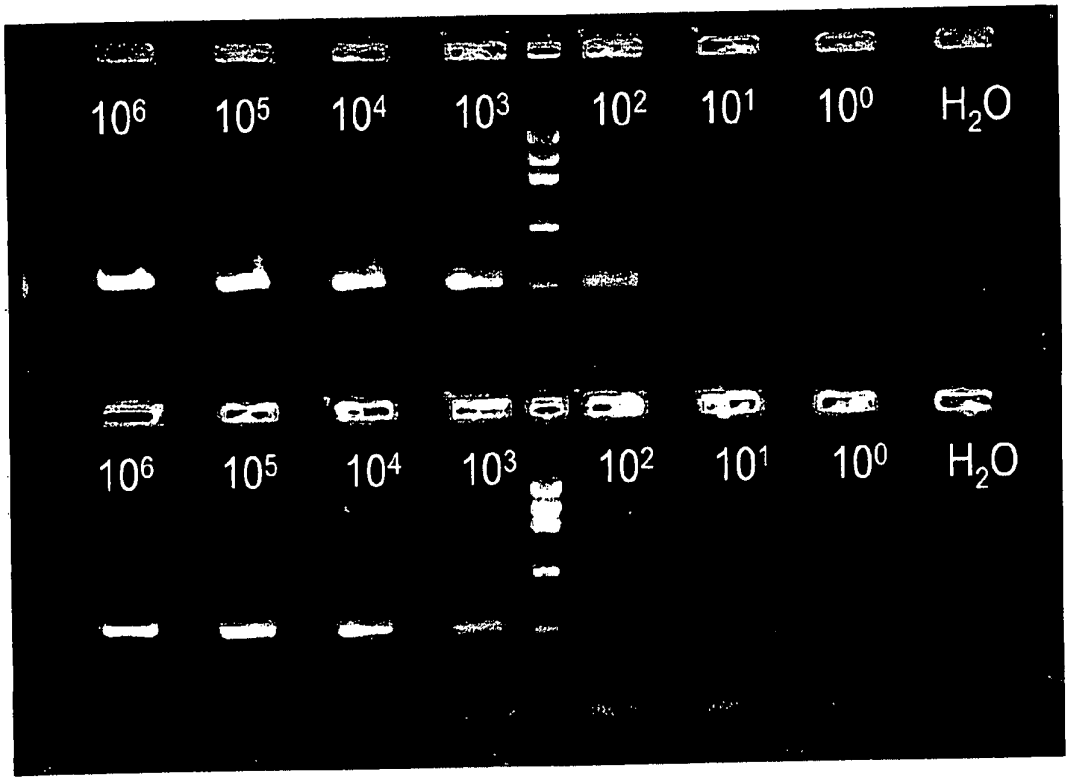
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P33-4
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P2Fwd-10/
P33+

SEQUENCE LISTING

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Ebersole, Richard C.
Schwartz, Linda J.
Jackson, Raymond E.

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